RECIPROCAL BINDING OF SPHINGOSINE AND PHOSPHATIDIC ACID TO STEROIDOGENIC FACTOR 1 REGULATES THE TRANSCRIPTION OF CYP17

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RECIPROCAL BINDING OF SPHINGOSINE AND PHOSPHATIDIC ACID TO STEROIDOGENIC FACTOR 1 REGULATES THE TRANSCRIPTION CYP17

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This work is dedicated :

To Nikhil Urs, my beloved husband, without his love and support this journey would not have been possible. He always encouraged me to stay positive and keep smiling. I am so blessed to have such a wonderful and caring companion.

To Ashwini Bhonsle, my beloved mother, who taught me that I can accomplish anything with strong will and patience.

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LIST OF SYMBOLS AND ABBREVIATIONS

SF1	Steroidogenic factor 1
Bt_2cAMP	Dibutryl cAMP
SPH	Sphingosine
PA	Phosphatidic acid
SPA	Scintillation Proximity Assay
IP	Immunoprecipitation
DAGP	Diacylglycerol phosphate
lysoSM	Lyso-Sphingomyelin
PIP2	Phosphatidylinositol bisphosphate
PIP3	Phosphatidylinositol triphosphate
S1P	Sphingosine-1-phosphate
Cer1P	Ceramide-1-phosphate
DAGK	Diacylglycerol kinase
PPAR	Peroxisome proliferator-activated receptor
LXR	Liver X receptor
FXR	Farnesoid X receptor
SXR	Steroid X receptor
PXR	Pregnane X receptor
LRH	Liver receptor homolog
SHP	Small heterodimer protein
RAR	Retinoic acid receptor
VDR	Vitamin D receptor
CAR	Constitutive androstane receptor

SUMMARY

Steroidogenic factor (SF1) is an orphan nuclear receptor that is essential for steroid hormone-biosynthesis and endocrine development. Recent studies have demonstrated that phospholipids are ligands for SF1. In the present study our aim was to identify endogenous ligands for SF1 and characterize their functional significance in mediating cAMP-dependent transcription of human CYP17. Using mass spectrometry we show that in H295R adrenocortical cells SF1 is bound to sphingosine (SPH) under basal conditions and that cAMP stimulation decreases the amount of SPH bound to the receptor. We also show that silencing both acid and neutral ceramidases using siRNA induces CYP17 mRNA expression, suggesting that SPH acts as an inhibitory ligand. *In vitro* analysis of ligand binding using scintillation proximity assays show that several sphingolipids and phospholipids, including phosphatidic acid (PA), can compete with [^{3}H]SPH for binding to SF1, suggesting that SF1 may have more than one ligand and binding specificity may change with the changes in intracellular fluxes of phospholipids. Further, phosphatidic acid (PA) induces SF1-dependent transcription of CYP17 reporter constructs. Inhibition of diacyglycerol kinase (DAGK) activity using R59949 and silencing DAGK-θ expression attenuates SF1-dependent CYP17 transcriptional. We propose that PA is an activating ligand for SF1 and that cAMP-stimulated activation of SF1 takes place by displacement of SPH

INTRODUCTION

1.1 Theoretical Background on Steroidogenesis & Cytochrome P450 Enzymes

Steroid hormones are ubiquitous regulators of wide variety of physiological processes. They are derivatives of cholesterol and are synthesized in a variety of organs, including the adrenal gland and gonads. Steroid hormone biosynthesis requires a battery of oxidative enzymes located in both mitochondria and endoplasmic reticulum. These enzymes are members of the cytochrome P450 superfamily and steroid dehydrogenases (HSD) and are transcriptionally regulated by a cAMP/cAMP-dependent protein kinase (PKA) pathway.

P450 side chain cleavage enzyme (P450scc) is the enzyme that catalyzes the first and rate limiting step in steroidogenesis. Within mitochondria, P450scc converts cholesterol to pregnenolone [1, 2]. Pregnenolone itself is not a hormone, but is the precursor for the synthesis of all of the steroid hormones. Conversion of cholesterol to pregnenolone takes place in three successive monooxygenation reactions: 1) Hydroxylation of C-22, 2) hydroxylation of C-20, 3) cleavage of the C-20 & 22 bond. P450scc is expressed in all three zones of the adrenal cortex, in th leydig cells of testis, and in the theca interna of ovary In addition to steroidogenic tissues, the expression of P450scc has been detected in the brain, but at levels that are more than an order of magnitude lower.

3β hydroxysteroid dehydrogenase (3β-HSD) has two major catalytic activities which in concert, convert 3β-hydroxy-5-ene steroids into 3-keto-4-ene steroids[3]. In humans, rat and mouse there are at least 2 or 3 homologous genes encoding 3β-HSD, as

opposed to single genes encoding P450 enzymes. 3β-HSD is localized in the same steroidogenic cells as P450scc: in all three zones of the adrenal cortex, in the interstitial Leydig cells of the testis, in the theca interna and corpora lutea of the ovary[3].

P450c17 catalyzes two key reactions: 1) 17α-hydroxylation of C21 progesterone and pregnenolone, and 2) cleavage of the C17-C20 bond of C21 steroids[4]. The 17α-hydroxylation is a required step in glucocorticoid biosynthesis, whereas the C17-C20 bond side chain cleavage is essential for the biosynthesis of androgens. Immunohistochemical analysis of the adrenal gland has shown that P450c17 is present in the zona fasiculata and reticularis but not in zona glomerulosa [5]. This observation is consistent with the role of zona glomerulosa as the site of mineralocorticoid biosynthesis where 17α-hydroxylation is not a required activity. Like all steroidogenic genes, induction of the gene that encodes P450c17, CYP17, is mediated by cyclic AMP (cAMP). In human CYP17 gene, the homeo-domain protein Pbx 1 was shown to interact with protein kinase A in the cAMP-dependent regulation of CYP17 [6]. Further it has been demonstrated that transcriptional activation of human CYP17 in adrenal cells depends on complex formation among p54(nrb)/NonO, protein-associated splicing factor, and SF-1, which also participates in repression of transcription [7].

P450c21 enzyme is involved in the 21-hydroxylation of progesterone and 17-hydroxy-progesterone, yielding deoxycorticosterone and 11-deoxycortisol from the two substrates, respectively. The 21-hydroxylation step is an important step in the synthesis of gluco- and mineralocorticoids , and the deficiencies in these enzymes lead to salt wasting syndrome and congenital adrenal hyperplasia [8]. The major site of expression of P450c21 is the adrenal cortex, however low levels of P450c21 have been observed in

human lymphocytes and brain[9].Both P450c21 and P450c17 are regulated by adrenocorticotropin (ACTH) [10].

Conversion of testosterone into 17β-estradiol is catalyzed by aromatase (P450arom). In the ovary P450arom is expressed in granulosa cells, which is the major site of estrogen production in females [11]. However, this enzyme is widely expressed in many tissues such as breast, central nervous system, skin and placenta [12]. The gene encoding P450arom is the longest one among steroidogenic P450 genes, and is unique because it utilizes alternative promoters in a tissue specific manner.

17-hydoxy steroid dehydrogenase (17β-HSD), also referred as 17-keto-steroid reductase, catalyzes the reversible conversion of the 17-keto and 17β-hydroxy groups in androgens and estrogens, including androstenedione, dehydroepiandrosterone (DHEA), and 17β-estradiol [13]. This enzyme is expressed in the testis in Leydig cells of the interstitial tissue, and in the ovary, in theca interna, but not in granulosa cells or corpus luteum.

In the adrenal cortex, steroidogenesis in regulated both acutely (within minutes) and chronically (hours to days) by ACTH hormone. The chronic regulation is at the level of transcription of steroidogenic genes whereas acute regulation is mediated by facilitating the movement of cholesterol into the mitochondria. Both the acute and chronic responses occur via a cAMP-dependent protein kinase (PKA) pathway.

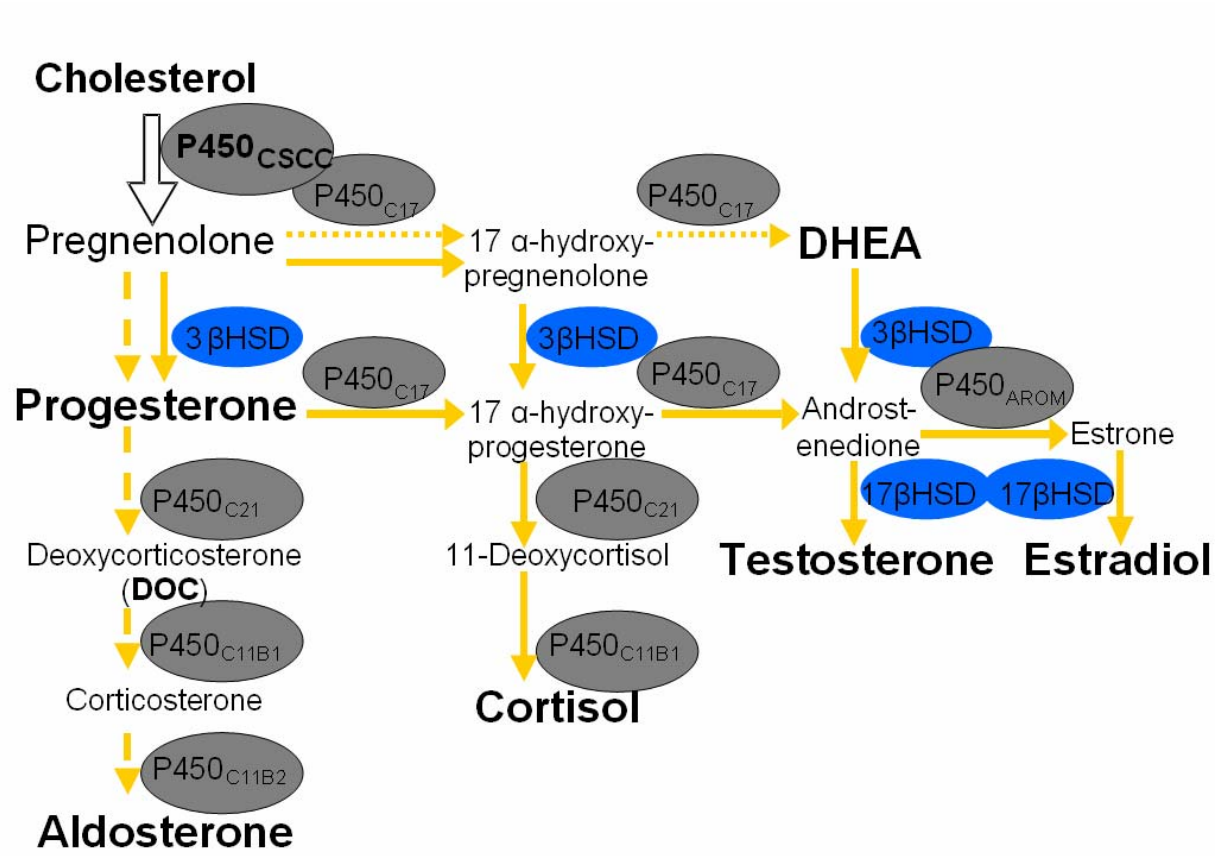

Figure 1: Steroidogenesis Pathway
This figure shows steps in steroidogenesis leading to production of Progesterone, Aldosterone, Cortisol, Testosterone and Estradiol. DHEA : Dehydroepiandrosterone, HSD: Hydroxy- steroid dehydrogenase.

1.2 Nuclear Receptors:

The nuclear receptor super family is an evolutionarily related group of transcription factors that regulate genes involved in a variety of physiological, developmental, and metabolic processes [14]. Nuclear receptors play roles in regulation of reproductive system by steroid hormones [15], control of development by thyroid and retinoid hormones and regulation of bile acid by cholesterol biosynthesis. Loss of nuclear receptor signaling, contributes to the development of endocrine related diseases such as breast cancer, ovarian cancer, prostrate cancer, diabetes and obesity.

1.21 Classification and Characterization of Nuclear Receptors

Steroid hormones were known to exist since the early 20^{th} century, but the concept of high affinity, tissue-specific, steroidophilic factors - receptors - as mediators of hormone function, gained credence from pioneering tissue-binding studies by Elwood Jensen, Jack Gorski, Anthony Means and other pioneers in the field [16]. By the end of the 1980's, the steroid and thyroid hormone receptors were first cloned and were found to exhibit extensive homology. This observation led to the discovery of other similarly structured receptors. Building upon biochemical evidence for the DNA binding and hormone binding activities of receptors, comparative alignment of the deduced amino acid sequences of the newly cloned receptor cDNAs indicated a modular organization of discrete motifs in each molecule, indicating a potential common ancestry and mode of action. As shown in figure 2, this superfamily contains not only ligand activated receptors, but large number of nuclear receptors that have been identified through sequence similarity to the known receptors, but have no identified ligand. These receptors are known as "orphan nuclear receptors". During recent years, specific ligands and also

target genes and physiological functions have been elucidated for many orphan nuclear receptors, and therefore are now called “adopted nuclear receptors” [17]. Many of these newly identified ligands are lipid molecules, and their nuclear receptors function as lipid sensors that respond to cellular lipid levels and elicit gene expression changes to maintain metabolic and structural homeostasis.

Although nuclear receptors are extremely diverse with regards to their ligands, they share common structural organization. The N-terminal region (A/B domain) is highly variable, and contains at least one constitutively active transactivation region (AF-1) and several autonomous transactivation domains (AD); A/B domains are variable in length, from less than 50 to more than 500 amino acids, and their 3D structure is not known. The most conserved region is the DNA-binding domain (DBD, C domain), which notably contains the P-box, a short motif responsible for DNA-binding specificity on sequences typically containing the AGGTCA motif, and is involved in dimerization of nuclear receptors. Between the DNA-binding and ligand binding domains is a less conserved region (D domain) that behaves as a flexible hinge between the C and E domains, and contains the nuclear localization signal (NLS), which may overlap on the C domain. The largest domain is the moderately conserved ligand-binding domain (LBD, E domain), whose secondary structure of 12 α-helixes is better conserved than the primary sequence [18].

	ENDOCRINE RECEPTORS	ADOPTED RECEPTORS	ORPHAN RECEPTORS
LIGANDS	High Affinity; Hormonal lipids	Low-Affinity; Dietary ligands	unknown
	ER-α,β	RXR α,β.γ	SF1
	PR	PPAR α,β.γ	LRH-1
	AR	LXR -α,β	DAX-1
	GR	FXR	SHP
	MR	PXR/SXR	TLX
	RAR α,β.γ	CAR	PNR
	TR α,β		NGFI-B α,β.γ
	VDR		ROR α,β.γ
	EcR		ERR α,β.γ
			RVR α,β.γ
			GCNF
			TR 2,4
			HNF-4
			COUP-TF α,β.γ

Figure 2: Classification of Nuclear receptor
Nuclear receptors can be subdivided into three or four groups, depending on the source and type of their ligand. Receptors with known physiological ligands are shown in color, and current orphan receptors are shown in gray. The first group is represented by classic nuclear steroid hormone receptors. Members of this group include the glucocorticoid (GR), mineralocorticoid (MR), estrogen (ER), androgen (AR), and progesterone (PR) receptors. The second nuclear receptor paradigm is represented by the adopted orphan nuclear receptors that function as heterodimers with the retinoid X receptor (RXR). Orphan receptors become adopted when they are shown to bind a physiological ligand. In contrast to the endocrine steroid receptors the adopted orphan receptors respond to dietary lipids. In addition to the adopted orphan receptors, there are four other RXR heterodimer receptors that do not fit precisely into either group. These include the thyroid hormone (TR), retinoic acid (RAR), vitamin D (VDR), and ecdysone (EcR) receptors. The ligands for these four receptors and the pathways they regulate employ elements of both the endocrine and lipid-sensing receptor pathways. The last group is of orphan nuclear receptors, whose ligands are unidentified.

Chawla AJ., Science Vo.l 294 1866-70

1.3 Steroidogenic Factor 1 (SF1)

SF1 is a member of the nuclear receptor family of proteins that share high homology with the Drosophila protein fushi tarazu factor-1 (FTZ-F1), a regulator of embryogenesis and metamorphosis [19]. SF1 is classified as a nuclear receptor on the basis of its structural homology and functional similarity with many members of this family of proteins. It has a zinc finger DNA binding domain, an intervening hinge region, and a carboxy-terminal putative ligand binding domain (LBD). However, SF1 belongs to the subgroup of nuclear receptors that are distinguishable for three reasons: 1) SF1 differs structurally from most other nuclear receptors, as it lacks an amino-terminal (A–B) domain, 2) unlike other nuclear receptors SF1 binds to its cognate DNA response element as a monomer, 3) SF1 belongs to the subgroup of nuclear receptors that are termed orphan nuclear receptors due to the absence of a *bona fide* ligand which can activate SF1 dependant gene transcription [20]. Increased expression of SF1 in steroidogenic cells and its very high homology to Drosophilla protein FTZ-F1 indicates the role of SF1 as an important regulator in mammalian steroidogenesis.

Studies carried out on SF1 knock out mice indicate that SF1 plays an important role in reproductive development (Figure 4). Studies using SF1+/- mice have established that SF1 is not essential for prenatal survival, as SF1 haplo-insufficient mice are born at a normal Mendelian frequency. SF1 null mice have neither adrenal glands, nor male or female gonads, since adrenocortical and gonadal precursor cells assemble in the urogenital ridge before embryonic day 12 and they subsequently undergo apoptosis [21] .

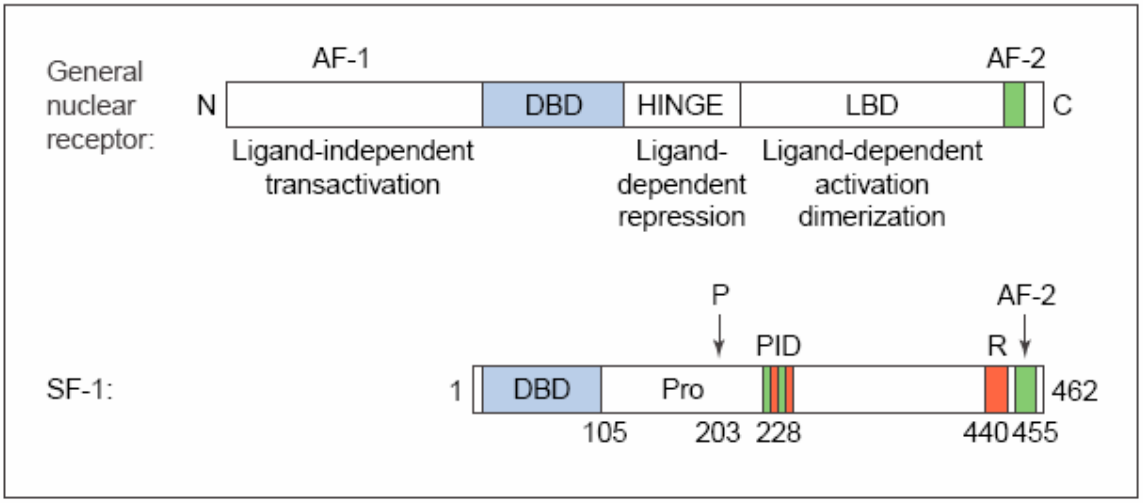

Figure 3: The structure of steroidogenic factor 1 (SF1), compared with the general structure of nuclear receptors
In addition to the shared DNA-binding domain (DBD), most receptors contain two activation function (AF) domains. SF1 lacks a functional AF-1, and its transcriptional activation function resides within its C-terminal region, which harbours the conserved AF-2 hexamer. A repression (R) domain is adjacent to the AF-2 hexamer, and a proximal interaction domain (PID) is located closer to the DBD. Serine-203 residue is a target for phosphorylation (P) by MAP kinases. A region C-terminal to the DBD, which includes proline-rich (Pro) sequences, mediates interaction with transcription factor IIB (TFIIB) and c-Jun.

Sadovsky Y. et al.: *Reviews* of Reproduction, 136–142, 2000

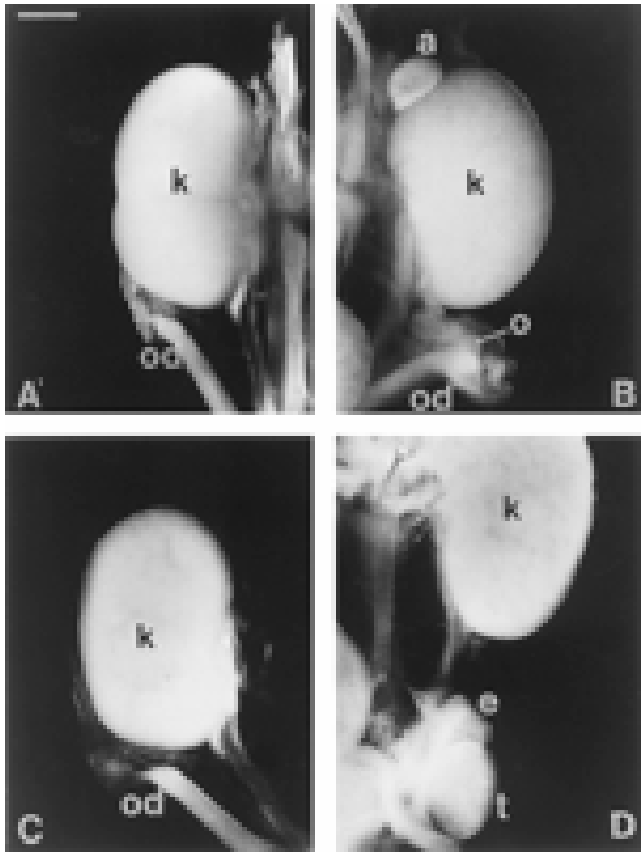

Figure 4: Phenotype of SF1 Knock out mice.

A. SF1 -/- female mouse lacks adrenal glands and ovary.
B. SF1 +/+ female mouse with normal adrenal gland and oviduct.
C. SF1 -/- male mouse lacks epididymis and testis.
D. SF1 +/+ male mouse with normal epididymis and testis.

X. Luo *et al*.: *Cell* 77:481–490, 1994

As gonads fail to develop in SF1 null mice, genotypically SF1 null mice exhibit sex reversal. Studies showing the diminished expression of β̃subunit of Leutinizing Hormone and follicle stimulating hormone in SF1 -/- mice demonstrate that SF1 is critical component of endocrine tissue development [21, 22].

SF1 regulates the expression of all genes that encode steroidogenic enzymes and other regulators of endocrine function.

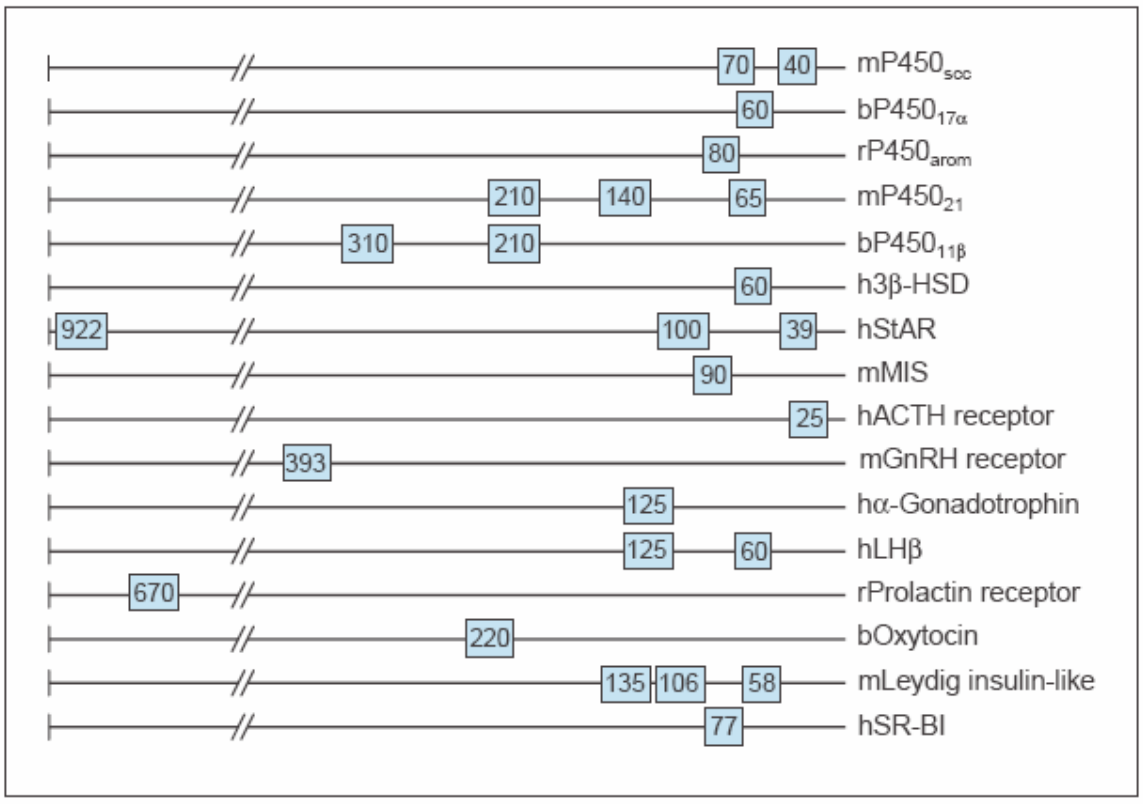

Figure 5: Promoter elements that mediate the effect of steroidogenic factor 1 (SF1) on important endocrine and reproductive targets.
HSD: hydroxyl-steroid dehydrogenase; StAR: steroidogenic acute regulatory protein; MIS: Müllerian inhibitory substance; ACTH: adrenocorticotrophic hormone; SR-BI: scavenger receptor B-1. LH: Leutinizing hormone, m: mouse, b: bovine, h: human, r: rat

A B

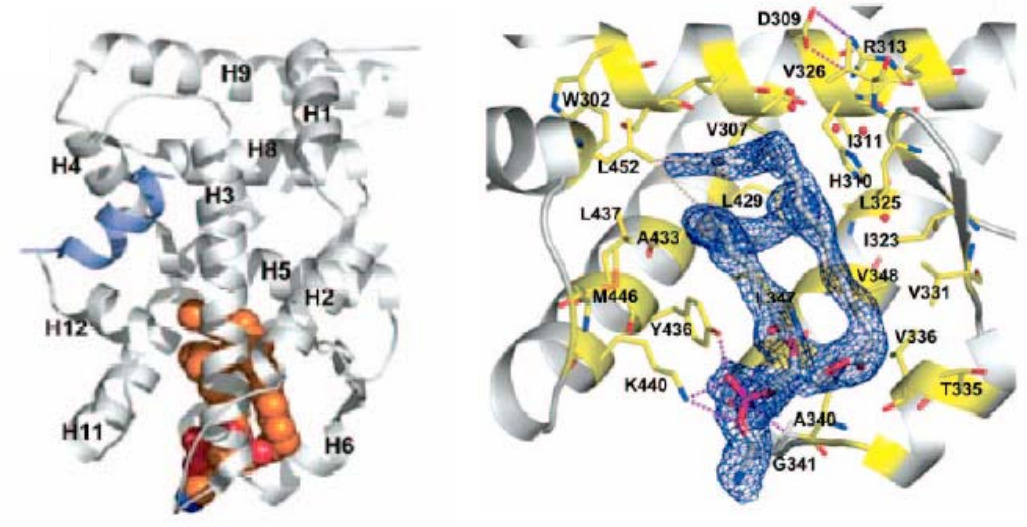

Figure 6: The human SF1 (hSF1) structure complexed with phospholipids (A & B) Residues of the hSF1 ligand-binding pocket (stick models colored yellow and by atom type),showing salt bridge and hydrogen bonds(dotted lines) to the Phosphatidyl ethanolamine (PE)(stick Models colored white and by atom type).

These studies led the researchers to postulate that SF1 activates gene expression in a ligand independent manner. However, recent x-ray crystallographic studies (Fig 6) carried out independently by three laboratories have shown that phospholipids are ligands for SF1 [23-25]. Specifically, Krylova, *et al.*, have demonstrated that phosphatidyl inositols specifically interact with the ligand binding domain (LBD) of SF1 and that ligand binding is required for maximal activity of the receptor [23]. Li *et al.* found that receptor was shown to have a large (approximately 1600 Å) LBD that interacts with phospholipids that have fatty acid side chains between twelve and eighteen carbons [24].

Moreover, the conformation established by the ethanolamine moiety on the phosphatidyl ethanolamine (PE) identified suggested that phospholipids with other head groups might also bind to SF1 [24]. Significantly, the large binding pocket led the authors to speculate that SF1 may readily exchange its ligands to respond to different phospholipids species [24].

The central hypothesis of the present study was to identify endogenous ligands bound to SF1and characterize the functional significance of these molecules in mediating cAMP-dependent transcription of human CYP17 in the adrenal cortex. To test this hypothesis, mass spectrometry was used to identify molecules bound to SF1 purified from control or cAMP-stimulated H295R human adrenocortical cells. The effect of various lipids on SF1 was examined using a reporter gene assay and real time RT-PCR. Site- directed mutagenesis was used to mutate amino acids in the LBD of SF1 and these mutants were used to determine amino acids critical for ligand binding. Finally RNA interference (RNAi) was used to modulate cellular pools of ligand, in order to determine the effect on the ability of SF1 to activate CYP17 transcription.

CHAPTER2

MATERIALS AND METHODS

2.1 Reagents.

Dibutyryl cAMP (Bt_2cAMP) was obtained from Sigma (St. Louis, MO). Phospholipids and sphingolipids were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). PA (egg yolk) was dissolved in chloroform, dried under nitrogen gas and resuspended in 50 mM Tris-HCl, pH 7.4 by ultrasonication. The diacylglycerol kinase inhibitor 3-2,3-dihydro-2-thioxo-4(1H)quinazolinone (R59949) [26] was obtained from EMD Biosciences, Inc. (La Jolla, CA).

2.2 Cell Culture.

H295R adrenocortical cells [27, 28] were generously donated by Dr. William E. Rainey (University of Texas Southwestern Medical Center, Dallas, TX) and cultured in Dulbecco's modified Eagle's/F12 (DME/F12) medium (Invitrogen, Carlsbad, CA) supplemented with 10% Nu-Serum I (BD Biosciences, Palo Alto, CA), 0.5% ITS Plus (BD Biosciences, Palo Alto, CA), and antibiotics. Jeg3 human choriocarcinoma cells were obtained from Dr. Michael R. Waterman (Vanderbilt University School of Medicine, Nashville, TN) and cultured in DMEM medium containing 10% fetal bovine serum and antibiotics.

2.3 Immunoprecipitation.

For IP assays, H295R cells (150 mm dishes) were transfected with 45μg of mutant or wild type SF1-pCMV Tag1 per dish and treated with 1mM Bt_2cAMP for 4 h. Nuclear proteins were isolated using the NE-PER nuclear and cytoplasmic extraction reagent

(Pierce, Rockford, IL) and incubated with anti-SF1 (Upstate, Lake Placid, NY) or anti-FLAG M2 antibody (Stratagene, La Jolla, CA) and protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C with rotation. The mixture was then centrifuged, the supernatant removed, and the precipitant subjected to a series of 5-minutes washes. Agarose beads were washed three times with a modified RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin, and 10 μg/ml leupeptin) and five times with PBS. The samples were then analyzed by mass spectrometry for detection of lipids and by SDS-PAGE (10% gel) and Western blotting to confirm SF1 expression.

2.4 Analysis of sphingolipid and phospholipid molecular species.

For sphingolipid measurements, immunoprecipitated SF1 was analyzed by liquid chromatography, electrospray ionization, tandem mass spectrometry (LC-ESI-MS/MS) as described previously [29, 30]. The internal standards for quantifying the amount of sphingolipid species bound to the receptor were obtained from Avanti Polar Lipids (Alabaster, AL).Briefly 0.5ml of methanol and 0.25ml of chloroform with internal standards was added to the immunoprecipitated SF1. Internal standards include 0.5nmol of each C12-SM, C12-Cer, C12-GlcCer, C12-LacCer, C20-SPH, C20-Sa, C17-Sphingosine 1-phosphate, & C17-Sphinganine-phosphate. The pellet was sonicated and incubated overnight at 48ºC in a heating block. The sample was cooled and 75μl of 1M KOH in methanol was added, sonicated and incubated for 2h at 37ºC. Four hundred micro liter of sample was saved for MS/MS analysis of sphingoid bases, sphingoid 1-P, and Cer-P. Remaining sample was neutralized with 3μl of glacial acetic acid. After adding 1ml of chloroform and 2ml of water saple was mixed well and centrifuged. The upper layer was removed leaving the interface. The solvent and water was removed form

the lower layer using speed vac and MS/MS s was carried out for complex sphingolipids in this fraction. The sample saved for sphingoid bases was dried in the same way. The samples were loaded onto appropriate HPLC columns that are directly coupled to the ion source of a tandem mass spectrometer. The mass spectrometer was programmed to monitor specific, individually optimized precursor and product ion pairs in specific time frames. The signal generated by each ion transition uniquely identifies molecular species by retention time, mass, and structure. Furthermore, quantification was achieved by direct correlation of the peak areas generated by the internal standards with those of the endogenous species. Ionization and dissociation (collision energy) conditions were optimized for individual molecular species.

2.5 Molecular modeling.

Docking of Sphingosine (SPH) and Phosphatidic acid (PA) (34:1 and 36:1) into the ligand binding pocket of the published crystal structure of SF1 [24] was done using the INSIGHTII module of AFFINITY (Accelrys, San Diego, CA. A general method was developed for docking ligands into the binding pocket of the SF1 LBD crystal structure. After obtaining the coordinates of the crystal structure from the Protein Databank, it was prepared for docking. First SF1 LBD and the its associated ligand PE were unmerged. Hydrogen atoms were added to the entire SF1 LBD using a pH of 7.4 to determine the charge state of amino acid side chains. The protein was "soaked" in a 5 Å layer of water so that the docking is performed in explicit solvent. Soaking is accomplished by placing the protein in the center of an equilibrated box of water followed by removing water molecules that either contact atoms in the protein or are further than 5 Å from the protein. *Affinity* applies molecular mechanics in searching for and evaluating docked structures. In order to make the search fast enough for practical applications, the ligand/receptor system

is partitioned into “bulk” and “movable” atoms. Bulk atoms are defined as atoms of the receptor that are *not* in the defined binding site. These atoms are held rigid during the course of the docking search. Movable atoms consist of atoms in the binding site of the receptor and ligand atoms. These atoms can move freely, except for binding site atoms close to bulk atoms. For docking, the ligand is manually placed in the binding site of the protein by superimposing atoms of the ligand to be docked onto atoms of the crystallographic PE. The amino acids within 5 Å of the placed ligand are defined as the “binding site”; these are allowed to move during the docking process. You can define the binding site as residues which are within 5Å of the ligand, if the ligand is already in the binding site or if you are studying docking of a novel ligand whose binding site to the receptor is unknown, you can use the binding site of a known ligand for the new ligand. Hydrogen bond donors and acceptor atoms are defined for both the ligand and atoms within 5 Å of the placed ligand. Torsions are also defined for the ligand. A Monte-Carlo simulation is used to generate possible binding modes of the ligand-protein pair. The ligand is randomly translated and rotated in the x, y, and z dimensions and the torsions are rotated to generate a new, random ligand placement. The resulting structure is minimized and the energy of the new structure is compared to the previous structure. If the energy of the new structure is lower than the energy of the previous structure, the new structure is accepted. If the energy of the new structure is greater than the energy of the previous structure, it will be accepted if the energy is within a predefined energy range. The final step is to calculate the ligand-receptor interaction energies for each accepted and minimized structure. Discover is used to calculate the van der Waals and electrostatic energies. These energies can be used to compare the docked structures.

2.6 Bacterial expression of SF1.

PCR was used to add 6 histidine residues to the LBD of SF1 and the PCR product cloned into the pET17b vector (Novagen, Madison, WI). The construct was transformed into BL21 cells and the cultures grown until the OD_{600nm} equaled 0.4. Isopropyl-beta-D-thiogalactopyranoside (0.4 mM) was added and the cultures grown for 6 h at 28°C. His-tagged SF1 was purified using nickel affinity chromatography using a His-Bind purification kit (Novagen, Madison, WI). Expression was verified by SDS-PAGE and MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry.

2.7 Scintillation proximity binding assay.

Scintillation proximity assays [31, 32] were carried out using purified and biotinylated SF1. SF1 (500 nM) was biotinylated using a EZ-Link Sulfo-NHS-LC-biotinylation kit (Pierce, Rockford,IL) for two hours at 4ºC, with rotation. The reaction was desalted to remove non-reacted biotinylation reagent as per the manufacturer's instructions. Streptavidin-PVT SPA beads (Amersham Biosciences, Piscataway, NJ) were suspended in assay buffer (50 mM Tris-Cl, pH 8.0, 50 mM KCl, 1 mM EDTA, 1 mM CHAPS, 1 mM DTT, 0.1 mg/ml BSA) at 0.5 mg/ml. The biotinylated receptor was added to a final concentration of 50 nM and the mixture incubated for four hours at 4ºC, with rotation.

The reaction was centrifuged at 4,000 rpm for 10 minutes and the uncoupled receptor (supernatant) was removed. The beads were resuspended in assay buffer, incubated for 15 minutes and centrifuged again. Beads were resuspended in 1 ml assay buffer and stored at 4ºC until use. For competition experiments, lipids obtained from Avanti Polar

Lipids (Alabaster, AL) were added at concentrations ranging from 0.1nM to 100 μM to reactions containing 10 nM [^{3}H]-SPH. Vials were incubated for 2 h at room temperature followed by scintillation counting. All assays were performed at least three times in triplicate.

2.8 Transient transfection and reporter gene analysis.

To determine effect of mutations in the LBD on SF1-dependent transcriptional activity Jeg3 human choriocarcinoma cells (lack endogenous SF1)were sub-cultured onto 12-well plates and 24 h later transfected with 250 ng of hCYP17 57-pGL3 reporter plasmid [33] and 100 ng of wild type or mutant (H310A, G341K, Y436A, K440D) SF1-pCMV Tag1 (Invitrogen, Carlsbad, CA) using GeneJuice (Novagen, Madison, WI). Wild type SF1 was obtained from Ken-ichiro Morohashi, (National Institute for Basic Biology, Okazaki, Japan) and cloned into the pCMV Tag1 vector. Mutants were prepared by using a QuikChange site-directed mutagenesis kit (Stratagene,La Jolla, CA) and were confirmed by sequencing. Cells were co-transfected with 10 ng of the Renilla luciferase plasmid (phRL, Promega, Madison, WI) for normalization. Twenty-four h after transfection the cells were treated with 1 mM Bt_2cAMP for 6 h and the transcriptional activity of the CYP17 reporter gene determined using a dual luciferase assay (Promega, Madison, WI).

To examine the effect of sphingolipids and phospholipids on SF1-dependent CYP17 reporter gene activity, Jeg3 human adrenocortical cells were transfected with 250 ng CYP17 57-pGL3, 100 ng SF1-pCMV Tag1, and 10 ng of the Renilla luciferase plasmid (for normalization). Twenty-four hours after transfection cells were treated for 6 h with 1 mM Bt_2cAMP or PA (1 nM to 1 μM). The role of DAGK in cAMP-dependent CYP17

transcription was examined by transfecting H295R cells with 250 ng CYP17 57-pGL3 for 24 h followed by treatment with 1 mM Bt_2cAMP and R59949 (DAGK inhibitor) for 6 h and harvested for dual luciferase assays.

2.9 RNA interference and Real Time RT-PCR.

Cells were sub-cultured into 12-well plates and 24 h later transfected with 50- or 150 nM of small interfering RNAs (siRNAs) directed against acid ceramidase (ASAH1), neutral ceramidase (ASAH2), and alkaline ceramidase (ASAH3), diacylglycerol kinase-alpha (DAGK-α), diacylglycerol kinase-zeta (DAGK-ζ), diacylglycerol kinase-theta (DAGK-θ), phosphoinositide 3-kinase (PI3K), or PTEN, (Dharmacon, Lafayatte, CO) using siIMPORTER (Upstate, Lake Placid, NY). The cells were incubated for 72 h then treated with 1 mM Bt_2cAMP for 12 h.

Expression of CYP17 was determined by real time RT-PCR. For quantitative RT-PCR, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) and amplified using the iScript One-Step RT-PCR Kit (Bio-Rad, Hercules, CA) on an iCycler real-time thermocycler (Bio-Rad, Hercules, CA). TaqMan probes (Applied Biosystems, Foster City, CA) were used to detect CYP17. PCR reactions were as follows: 1) 1 X 94ºC, 5 minutes, 2) 45 X 95ºC, 30 seconds, seconds, 52ºC, 45 seconds. CYP17 expression was normalized to β-actin (forward 5'-CGGCTCCGGCATGTGCAAG-3' and reverse 5'-TGACGATGCCGTGCTGCATG-3') and calculated using the delta-delta cycle threshold (ΔΔCT) method.

CHAPTER 3

RESULTS

3.1 SF1 binds to sphingolipids under basal conditions.

We carried out mass spectrometric analysis to identify sphingolipids bound to SF1 that was immunoprecipitated from control or cAMP-stimulated H295R cells. Cells were transfected with SF1-pCMV Tag1, treated with Bt_2cAMP and immunoprecipitated with an anti-SF1 antibody for analysis by LC-ESI-MS/MS. As shown in Figure 7, LC-ESI-MS/MS analysis revealed that SPH and lyso sphingomyelin (lysoSM) are bound to SF1 isolated from control cells. When treated for 4 h with 1 mM Bt_2cAMP, the amount of SPH and lysoSM decreases by 44% and 36%, respectively. These findings suggest that SPH and/or lysoSM bind to SF1 and maintain the receptor in an inactive conformation and that cAMP stimulation decreases sphingolipid binding, thereby activating the receptor. The amounts of other sphingolipids (ceramides, sphingomyelin, sphinganine, S1P, ceramide-1-Phosphates) were less than 15 pmol/mg total protein.

3.2 SPH and lysoSM bind SF1 in vitro

Because LC-ESI-MS/MS analysis of SF1 immunoprecipitated from H295R cells does not allow us to determine the stoichiometry of sphingolipid-receptor binding, we performed the Scintillation proximity assay (SPA). This assay allows for analysis of the interaction between ligand and receptor and for estimation of relative binding affinities. The apparent equilibrium dissociation constant (K_i) for SPH and lysoSM was determined in competition binding assays. The beads were incubated with 10 nM [^{3}H]SPH in the presence of unlabeled SPH or lysoSM at concentrations ranging from 0.1 nM to 100 μM.

[^{3}H]SPH specifically bound to SF1 with a calculated K_i of approximately 360 nM, confirming LC-ESI-MS/MS studies and further demonstrating that [^{3}H]SPH binds directly to SF1. LysoSM on the other hand, had a K_i of approximately 1 μM, was not as effective in competing with [^{3}H]SPH for binding to SF1 (Figure 8)

3.3 Both sphingolipids and phospholipids compete with SPH for binding to SF1.

Since recent studies have shown that phospholipids are ligands for SF1 and that ligand binding is required for maximal activity [23, 24], we determined the ability of several phospholipids and sphingolipids to displace SPH using the scintillation proximity assay. As shown in Figure 9, most of the lipids tested displace [^{3}H]SPH from the receptor. Phosphatidylinositols (PIPs), which were bound to bacterially expressed SF1 used for crystallographic studies [23], showed varying abilities to decrease [^{3}H]SPH binding to the receptor. PI(3,4)P_2, PI(4,5)P_2, and PI(3,4,5)P_3 inhibited [^{3}H]SPH binding by 40%, 48%, and 57%, respectively, while PI(3,5)P_2 only decreased [^{3}H]SPH binding by 11%. PC (16:0-18:1) displaced 78% of [^{3}H]SPH, while PE (16:0-18:1) decreased [^{3}H]SPH binding to SF1 by 57%. PA (16:0-18:1) inhibited 82% of [^{3}H]SPH binding to SF1, while S1P displaced 93% of labeled SPH

3.4 Silencing ceramidase induces CYP17 transcription.

Based on the SPA competition assays demonstrating that SPH binds to SF1 with a higher affinity than lysoSM, we carried out studies to further characterize the role of SPH in SF1-dependant CYP17 transcription. SPH is produced when ceramidases cleave the amide bond in ceramide. To date, three types of ceramidases have been described and are classified as acid (ASAH1), neutral (ASAH2) and alkaline (ASAH3) according to the pH at which optimal activity is achieved [34-37]. Since we have shown that cAMP

decreases the amount of SPH bound to SF1, we hypothesized that decreasing the cellular production of SPH would increase CYP17 gene expression. To test this hypothesis, H295R cells were transfected with siRNAs directed against ASAH1, ASAH2, and ASAH3 and the mRNA expression of CYP17 was measured by real time RT-PCR. As shown in Figure 10, silencing ASAH1 and ASAH2 increased CYP17 mRNA expression by 4.1- and 2.0-fold, respectively, further supporting a role for SPH as an inhibitory ligand for SF1.

3.5 Phosphatidic acid activates SF1 transcriptional activity.

To determine if any of the lipids that displaced SPH in the scintillation proximity assay could activate SF1-dependent CYP17 reporter gene activity, Jeg 3 cells were transfected with the CYP17-57pGL3 plasmid and treated with varying concentrations of lipids. None of the sphingolipids tested were able to increase SF1-dependent reporter gene activity (Figure 11). Jeg3 cells were used because they lack endogenous SF1. As shown in Figure 12, PA treatment resulted in a dose-dependent increase in CYP17 transcriptional activity which was maximal at 100 nM. Based on LC-ESI-MS/MS data demonstrating that cAMP treatment decreased the amount of SPH bound to SF1, we hypothesized that SPH may act as an antagonist and inhibit the ability of PA to activate SF1-dependent CYP17 reporter gene activity. As shown in **Figure 12**B, increasing concentrations (1- to 25 μM) of SPH attenuated the stimulatory effect of PA on CYP17 transcriptional activity.

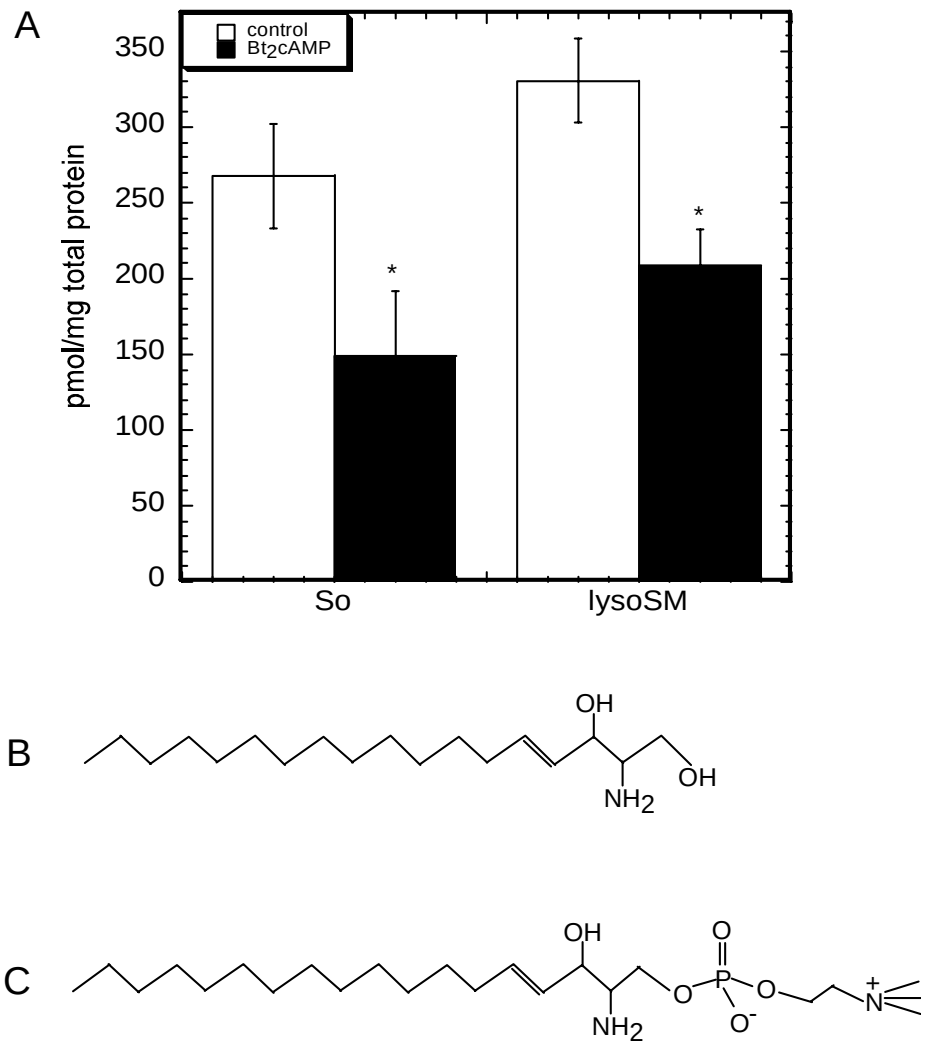

Figure 7: SPH & lysoSM binds to SF1 *in vivo*.
A, Quantification of LC-ESI-MS/MS analysis of SPH bound to SF1. H295R cells were transiently transfected with SF1-pCMVTag1 and treated for 4 h with 1mM Bt2cAMP. The lysates were immunoprecipitated with anti-SF1 antibody and the immunoprecipitated receptor analyzed by LC-ESI-MS/MS. Structures of SPH
(B) and lysoSM (C).

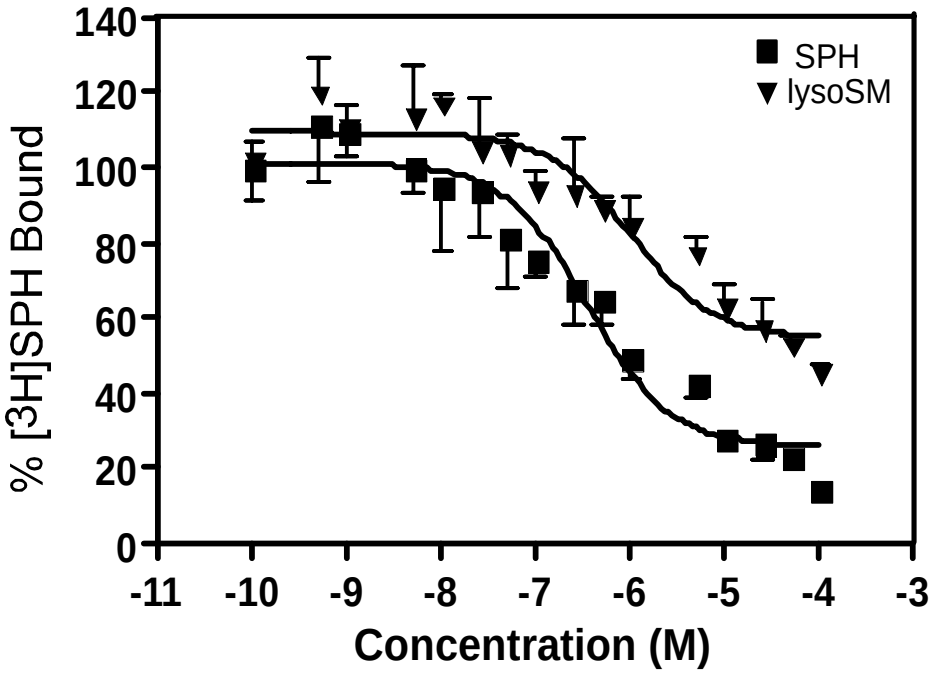

Figure 8: SPH binds to the LBD of SF1 *in vitro*.
His-tagged SF1 was expressed in *E. coli*, purified, biotinylated and coupled to streptavidin-coated SPA beads as described in Materials and Methods. Competition binding assay; Receptor-bound SPA beads were incubated with 10 nM [^{3}H] SPH and non-radiolabeled SPH (0.1 nM to 100 μM, filled squares) or lysoSM (0.1 nM to 100 μM, filled triangles). Data points represent the mean±SD of three assays performed in triplicate.

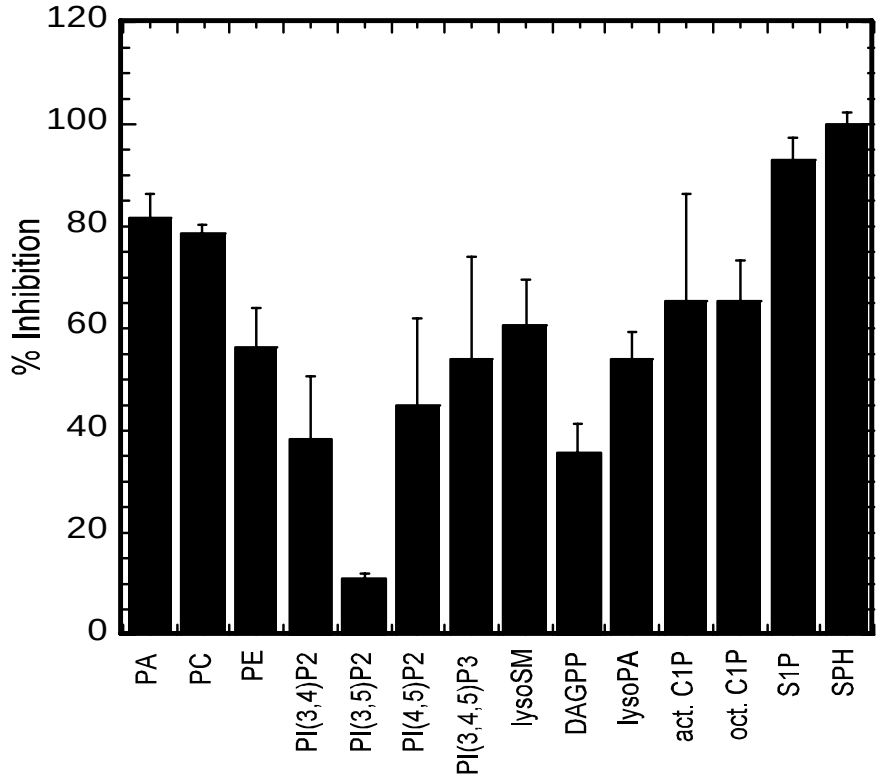

Figure 9: Phospholipids and sphingolipids compete with [3H]SPH for binding to SF1.

The LBD of SF1 was His-tagged, expressed in *E. coli*, biotinylated and coupled to streptavidin-coated SPA beads as described in Materials and Methods. Competition binding assays were performed with 10 nM [^{3}H]SPH and 10 μM of each lipid. Data represent the mean of three assays performed in triplicate + SD and are plotted as percent inhibition of [^{3}H]SPH binding where competition with unlabeled SPH is defined as 100%.

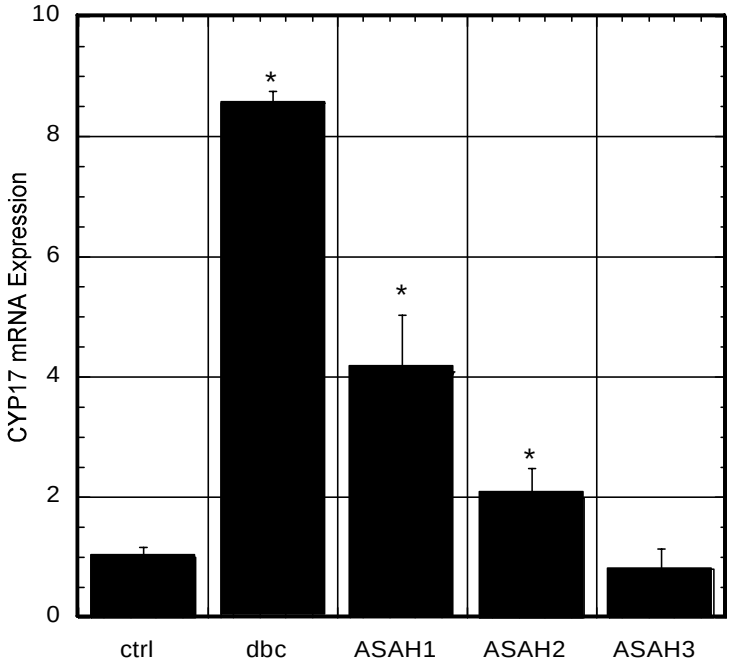

Figure 10: Silencing ceramidase expression induces CYP17 mRNA.
Cells were transfected with siRNA oligonucleotides targeted against ASAH1, ASAH2, or ASAH3 for 48 h. One group of untransfected cells was treated with 1 mM Bt2cAMP (dbc) for 12 h. Total RNA was isolated and CYP17 mRNA expression was determined using real time RT-PCR. Data graphed represents the mean + SEM of 3 separate experiments, each performed in quadruplicate. *, $p<0.5$ statistically different from untreated control.

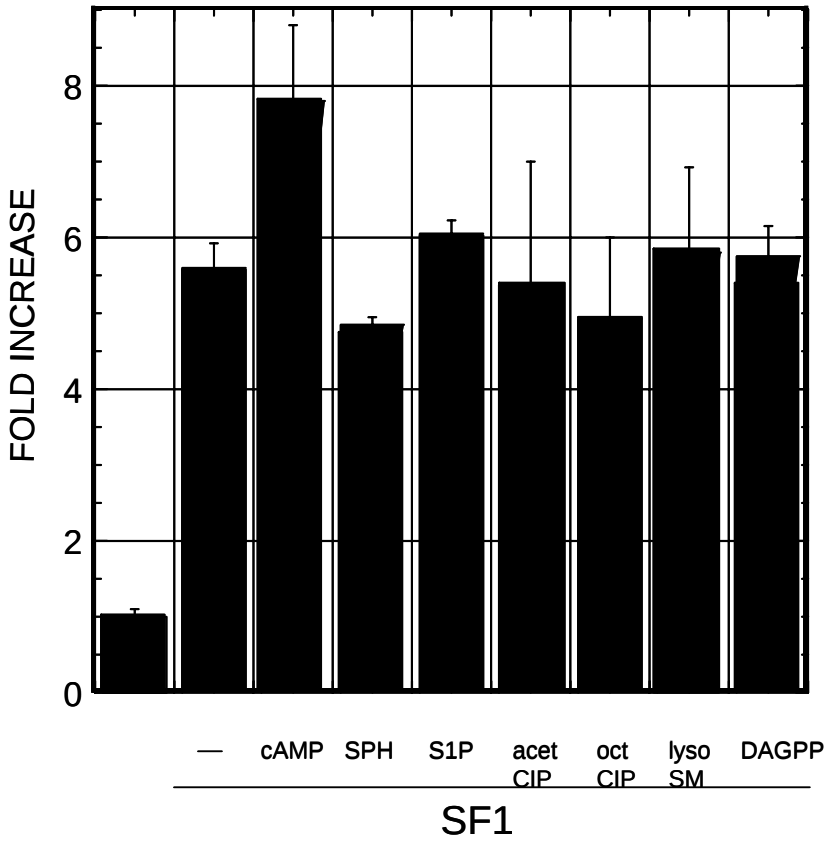

Figure 11: None of the sphingolipids inhibiting SPH binding in SPA, increase SF1 dependent reporter gene activity.
H295R Cells were transfected with 250 ng CYP1757-pGL3 and 50 ng of SF1-pCMVTag1 using Gene Juice. Twenty-four hours after transfection, the cells were treated with 10μM of all lipids except for S1P (1μM) for 6h. Data in the graph is normalized to Renilla activity and is expressed as fold increase over CYP17 57-pGL3. Data graphed represents the mean + SEM of at least 3 separate experiments, each performed in triplicate. *, $p<0.5$ statistically different from untreated control.

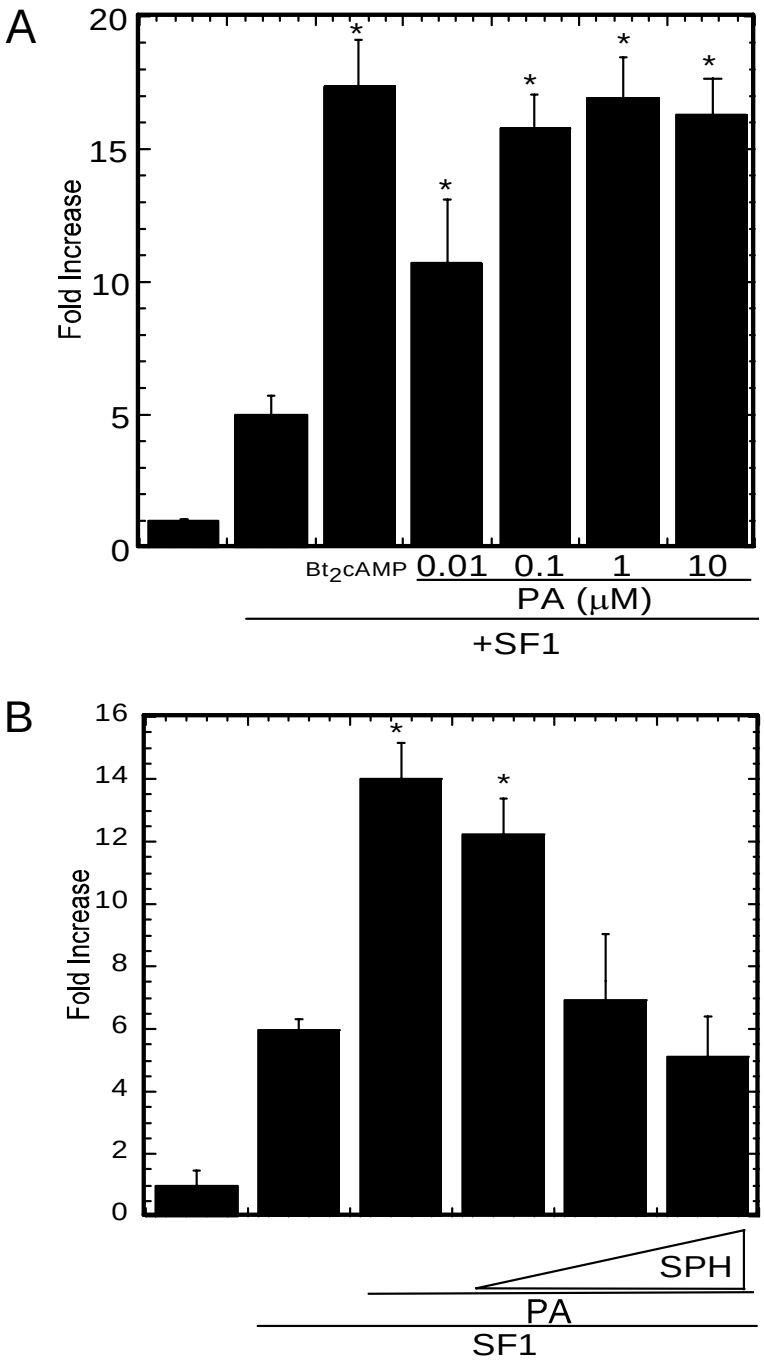

Figure 12: PA activates CYP17 transcriptional activity.
Jeg3 cells were transfected with CYP17 57-pGL3 and SF1-pCMVTag1, then treated with 1 mM Bt_2cAMP or PA (1 nM to 1000nM) for 6 h. B, Cells were transfected with 250 ng CYP17 57-pGL3 and 50 ng of SF1-pCMVTag1 using Gene Juice. Twenty-four hours after transfection, the cells were treated for 6h with 1 μM PA in the presence and absence of SPH (1-, 10- or 25 μM). Data in both graphs is normalized to Renilla activity and is expressed as fold increase over CYP17 57-pGL3. Data graphed represents the mean + SEM of 3 separate experiments, each performed in triplicate. *, $p<0.5$ statistically different from untreated control.

3.6 Effect of mutations in the LBD on CYP17-reporter gene activity.

We next characterized the effect of mutations in the LBD of SF1 on CYP17 reporter gene expression. Based on recent structural studies identifying residues that contact the ligand [23], we made constructs containing mutations in five amino acid residues (H310, G341, K440, Y436, and L452). Jeg3 cells (lack endogenous SF1) were transiently transfected with plasmids containing wild type or mutant SF1 and a plasmid containing the first 57 base pairs of the human CYP17 promoter fused to the luciferase gene as described previously [33]. Cells were treated for 6 h with 1 mM Bt_2cAMP or 10 nM PA. As shown in Figure 13, transfection of wild type SF1 resulted in a 5.2-fold increase in luciferase activity, which was further stimulated by Bt_2cAMP and PA. Mutations to histidine-310 and glycine-341 reduced SF1-dependent transcriptional activity by 29- and 36%, respectively. While mutations at Y436 and K440 completely attenuated the ability of SF1 to transactivate the CYP17 reporter construct (Figure 13). None of the mutant constructs were responsive to Bt_2cAMP or PA. Similar results were observed in H295R cells.

3.7 Mutations in the LBD alter SPH binding in vivo.

Both wild type and mutant FLAG-tagged constructs were transfected into H295R cells and immunoprecipitated for analysis by LC-ESI-MS/MS. Mutating histidine-310 to alanine decreased binding of SPH by 21% in control cells, however this mutation increased the binding of SPH in cAMP-treated cells compared to the wild type SF1 purified from cAMP-treated cells (Figure 14). Similar findings were observed with the G341A mutation. Interestingly, the K440A and Y436A mutants both exhibited

approximately a 70% decrease in binding to SPH in control cells (Figure 14). These observations suggest that K440 and Y436 are important for binding SPH and that H310 and G341 may play a role in the cAMP-stimulated exchange of SPH for a lipid whose binding is required for activation of the receptor. Of note, we have previously found that cAMP activates sphingolipid catabolism ([38]), thus it is possible that the cAMP-stimulated change in the cellular content of sphingolipids plays a role in activating SF1.

3.8 DAG kinase is required for SF1-/cAMP-dependent CYP17 transcription.

PA can be produced in several ways: phospholipase D (PLD) catalyzed hydrolysis of phosphatidylcholine, acylation of LPA, or phosphorylation of diacylglycerol by DAGK. To determine the effect of reducing cellular pools of PA, cells were treated with R59949 [26, 39, 40] to inhibit DAG kinase. As shown in Figure 15, R59949 attenuated the ability of both SF1 and cAMP to activate CYP17 transcription in a dose-dependent manner, suggesting that DAGK catalyzed formation of PA is required for synthesizing the activating ligand for SF1.

To determine if PLD catalyzed production of PA was important for SF1-dependent CYP17 transcription, transfected cells were also treated with 0.1% 1-butanol ([41]) for 2 h, however, this PLD inhibitor had no significant effect on the ability of SF1 to increase CYP17 luciferase activity (data not shown). There are nine mammalian isoforms of DAGK, most of which have been detected in the nucleus of various tissues and cell lines [42, 43]. To further characterize the role of DAG kinases in mediating cAMP-dependent CYP17 mRNA expression, H295R cells were transfected with siRNAs targeted at DAGK-θ. Seventy-two h after transfection, the cells were treated with 1 mM Bt_2cAMP for 12 h and CYP17 mRNA analyzed by real time RT-PCR. As shown in Figure 16,

silencing DAGK-θ expression inhibited the ability of Bt_2cAMP to stimulate CYP17 mRNA expression, while reducing the expression of DAGK-α or DAGK-ζ had no effect on cAMP-stimulated CYP17 transcription.

Because PI3K has been shown to regulate the activity of DAGK-θ ([44]), RNAi was used to examine the roles of PI3K and PTEN in cAMP-dependent CYP17 mRNA expression. As shown in Figure 18, silencing PI3K expression significantly inhibits cAMP-stimulated CYP17 mRNA expression. No role was found for PLDs in mediating cAMP-dependent CYP17 transcription (data not shown). These RNAi studies demonstrate that cAMP-dependent transcription of CYP17 in H295R cells requires the activation of both PI3K and DAG kinase-θ. Taken together, our studies indicate that cAMP activates CYP17 gene expression by promoting the exchange of an inhibitory lipid (SPH) for a stimulatory lipid (PA) in the ligand-binding pocket of SF1.

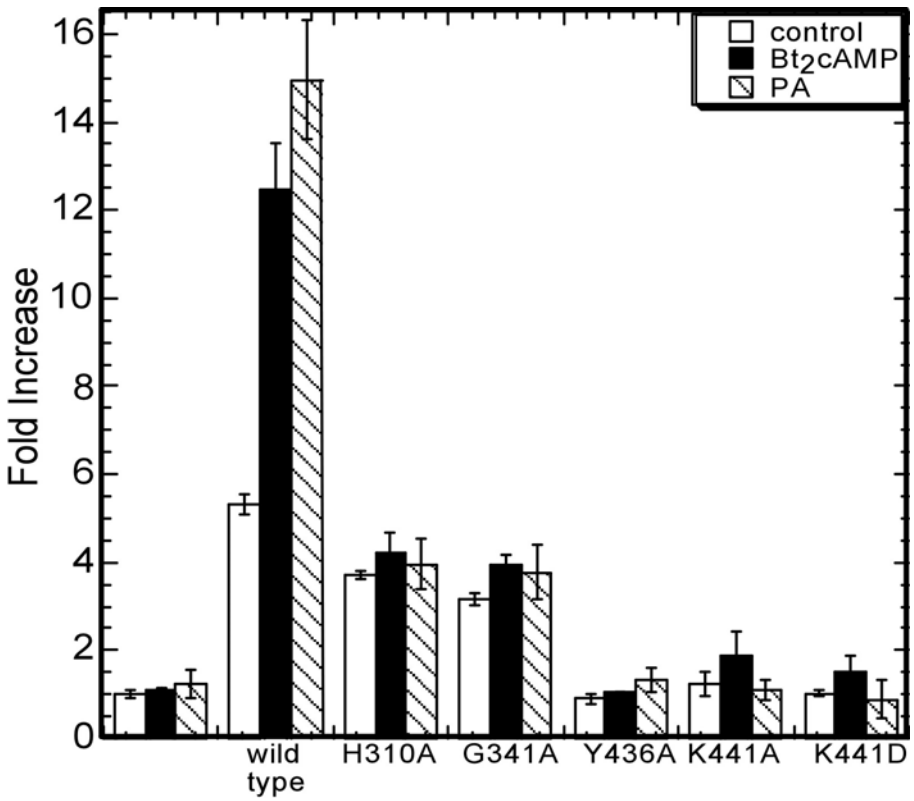

Figure 13: LBD mutants of SF1 show reduced transcriptional activity.
Jeg3 cells were transiently transfected with 250 ng CYP17 57-pGL3, and 100 ng wild type or mutant SF1 (in pCMV Tag1 vector) and treated with 1 mM Bt_2cAMP or 10 nM PA for 6 h. Data is normalized to the luciferase activity of the Renilla gene and is expressed as the fold increase over the CYP17 57- pGL3 plasmid. Data graphed represents the mean + the standard error from 4 separate experiments, each performed in triplicate. Data graphed represents the mean + SEM of 4 separate experiments, each performed in triplicate. * denotes statistically different from untreated wild type, $p<0.5$.

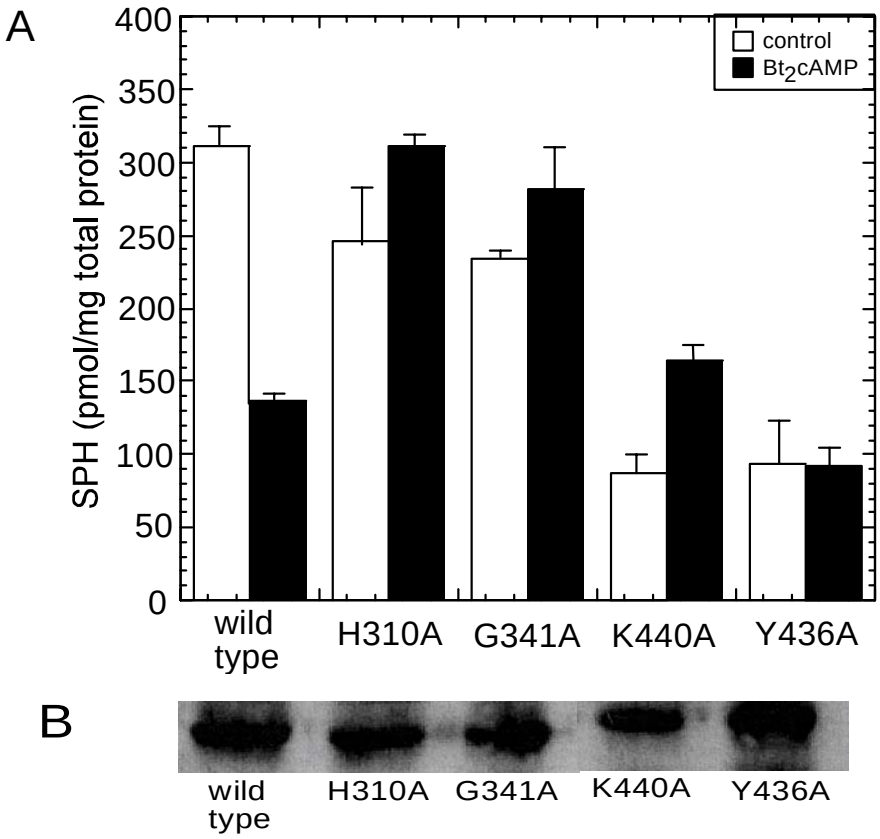

Figure 14: Effect of mutations in the LBD of SF1 on SPH binding in vivo.
A, Cells were transiently transfected with wild type or mutant SF1 and treated for 4 h with 1mM Bt2cAMP. A, The lysates were immunoprecipitated with anti-FLAG antibody and sphingolipid binding analyzed by LC-ESI-MS/MS. SPH amounts are expressed in pmol/mg total protein and determined by comparison to a standard. B, Expression of wild type and mutant receptor in transfected H295R cells. A fraction (10 µl of a 50% slurry) of the immunoprecipitated receptor was subjected to SDS-PAGE and Coomassie staining.

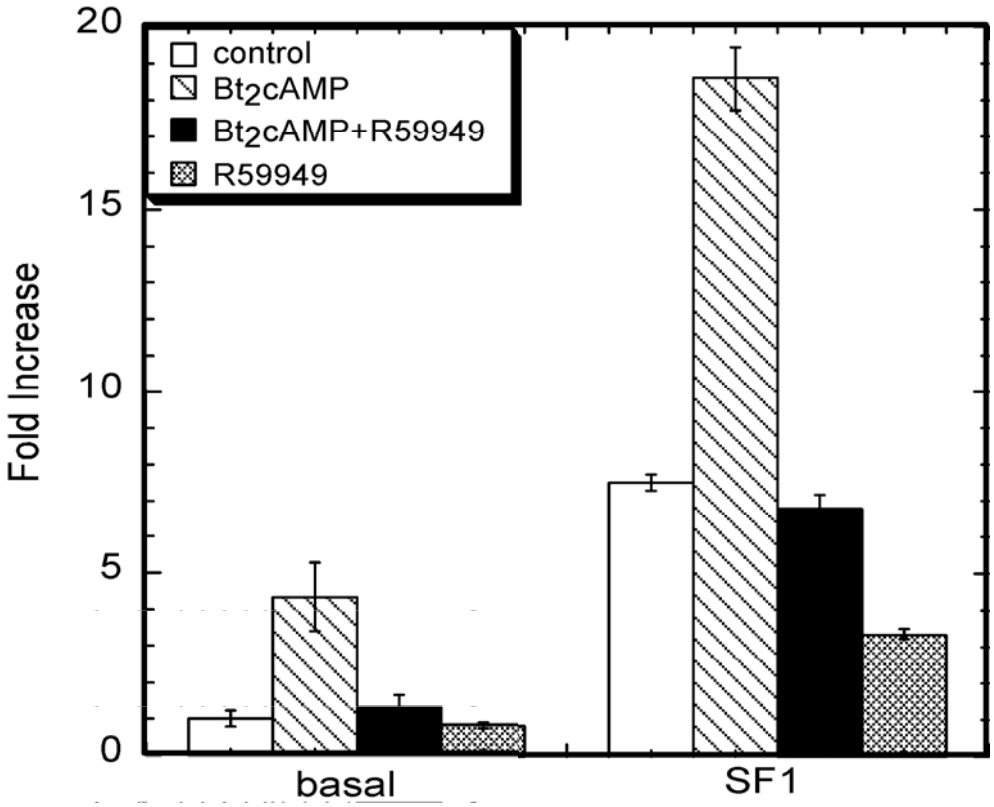

Figure 15: DAG kinase inhibition attenuates cAMP-stimulated CYP17 transcription activity.
H295R cells were transiently transfected with CYP17 57-pGL3 and SF1-pCMVTag1, then treated with Bt2cAMP (1 mM) in the presence and absence of R59949 (5 μM) for 6h.

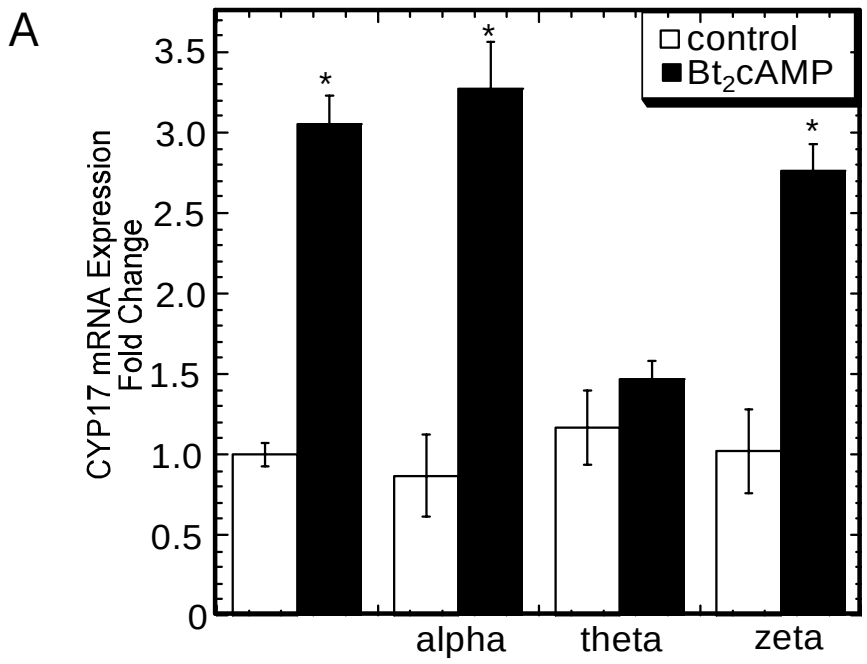

Figure 16: cAMP-dependent CYP17 transcription requires DAGK-θ.
Cells were transfected with 150 nM siRNAs directed against DAGK-α, DAGK-θ, or DAGK-ζ for 72 h, then treated with 1 mM Bt_2cAMP for 12 h. Total RNA was extracted and real time RT-PCR was performed. CYP17 mRNA expression was normalized to actin content. Values graphed represent the mean + SEM of 3 separate experiments, each performed in quadruplicate. *, $p<0.5$ statistically different from untreated control.

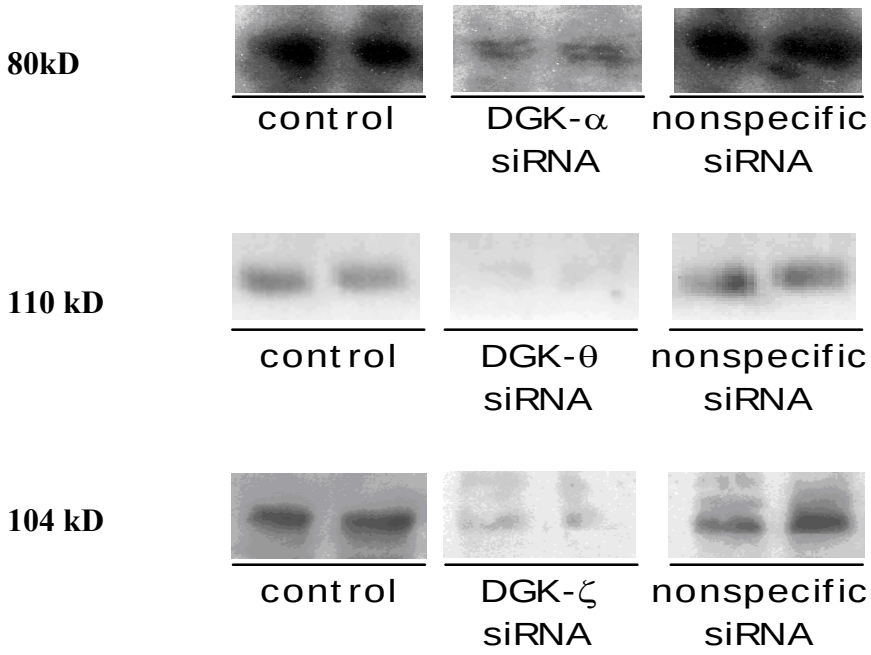

Figure 17: Effect of RNAi on DAGK protein expression.
Lysates from transfected cells were isolated and analyzed by SDS PAGE and Western blotting.

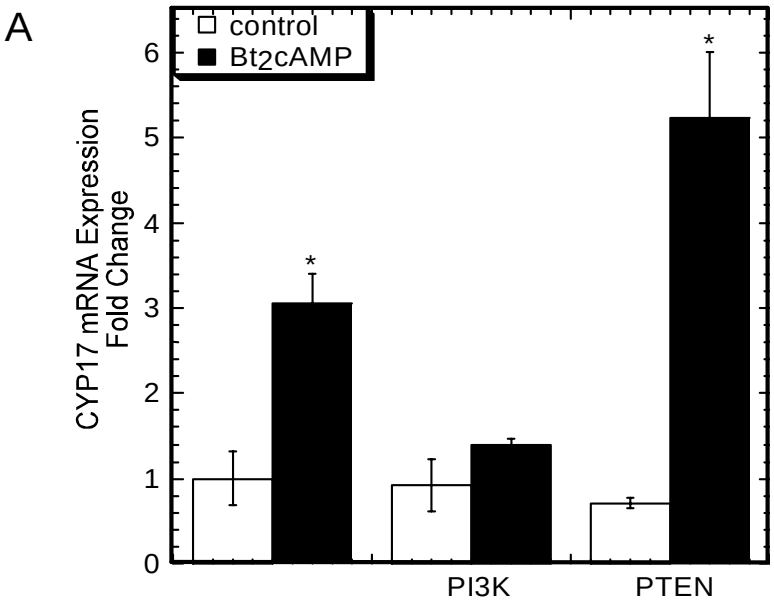

Figure 18: cAMP-stimulated CYP17 mRNA expression requires PI3K.
A, H295R cells were transfected with 150 nM siRNAs directed against PI3K or PTEN for 96 h, then treated with 1mM Bt_2cAMP for 12 h. Total RNA was extracted and real time RT-PCR performed to determine CYP17 mRNA expression. CYP17 mRNA expression was normalized to actin content. Values graphed represent the mean + SEM of at least 3 separate experiments, each performed in at least triplicate. *, $p<0.5$ statistically different from untreated control.

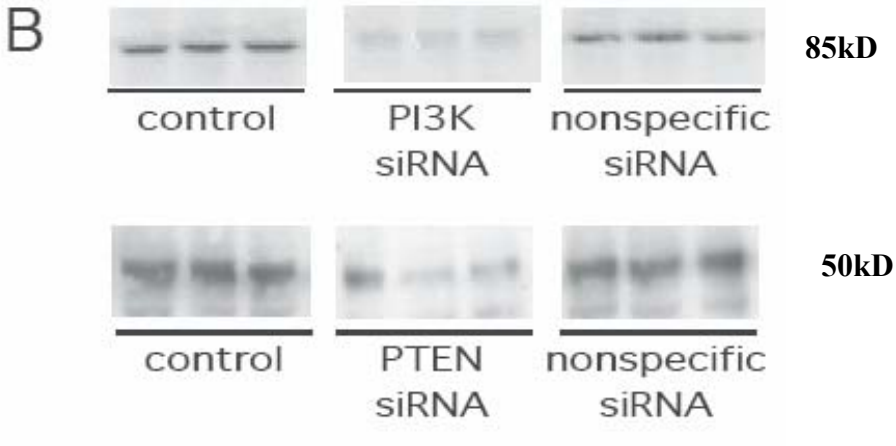

Figure 19: Knockdown of PI3K and PTEN protein expression using siRNA oligonucleotides.
Lysates from transfected cells were isolated and analyzed by SDS-PAGE and Western blotting.

CHAPTER 3

DISCUSSION

Nuclear receptors function as ligand activated transcription factors that regulate the expression of target genes to affect processes as diverse as reproduction, development, and general metabolism [45-48]. Orphan nuclear receptors, lack identified ligands, and hence provide a unique, and largely untapped, resource to discover new principles of physiologic homeostasis, and it is required for development of endocrine glands and sexual development [49, 50]. Several classes of lipids, including sterols, retinoids, fatty acids, and lipophilic xenobiotics, have been identified as ligands for orphan nuclear receptors [45]. Recently, crystallographic studies of bacterially expressed SF1 have identified another important class of lipids, phospholipids, as ligands for SF1 [23-25].

In the current study we used LC-ESI-MS/MS to identify ligands bound to SF1 endogenously expressed in H295R human adrenocortical cells and to characterize the effect of cAMP on the ability of these ligands to bind to SF1. SF1 is essential for steroid hormone biosynthesis and for the expression of steroidogenic genes. In the adrenal gland, steroidogenic gene transcription occurs in response to ACTH, a peptide hormone that binds to G protein-coupled receptors on the surface of adrenocortical cells and activates a cAMP signal transduction cascade [51]. Increased intracellular cAMP results in the activation of the PKA, and ultimately the coordinate increase in the transcription of steroidogenic genes. The direct target of PKA has yet to be determined, however several transcription factors, including SF1, are required for conveying cAMP-dependent transcription [51].

Mutation of A270W in murine SF1 reduces its ability to bind these phospholipids and the transcriptional activity of this ligand binding pocket mutant is also reduced as compared to wild type murine SF1 [23]. Mutagenesis studies carried out by Li *et al.*, in HEPG2 cells demonstrate that among amino acids L266W, A270W, L348W, V349W, A352W, A434W, K441E, the mutation K441E has a severe effect as it disrupts the specific hydrogen bonds with bound phospholipids [24]. Our reporter gene studies in Jeg3 cells, which lack endogenous SF1 (Figure 13) as well as in H295R human adrenocortical cells (data not shown) with SF1 containing mutations in the ligand binding pocket reveal that H310, G341, Y436, and K440 are critical for basal, cAMP, and PA-stimulated transactivation potential. Based on modeling studies, the reduction in activity observed with the K440 mutant is due to the inability of the phosphate moiety on PA to interact with the positively charged lysine. Glycine-341 is important for packing, thus increasing the size of this residue negatively impacts the ability of the receptor to bind PA. Based on the amount of SPH bound to the H310A and G341A mutants' immunoprecipitated from Bt_2cAMP-treated cells (Figure 14), we speculate that mutation of these two residues increases the stability of SPH in the binding pocket and prevents exchange of SPH for PA.

Our findings demonstrate that SF1 is a lipid binding protein. Specifically, we have shown that SPH is bound to SF1 immunoprecipitated from H295R cells (Figure 7). Importantly, the amount of SPH bound to SF1 is regulated by cAMP because SF1 purified from cells exposed to Bt_2cAMP contained decreased amounts of SPH. These findings suggest that SPH is an inhibitory ligand for SF1 and that activation of the receptor takes place by the exchange of SPH for an activating ligand. This hypothesis is

supported by siRNA data demonstrating that silencing the expression of acid and neutral ceramidases increases CYP17 mRNA expression (Figure 10). SPH is not synthesized *de novo* [52, 53], thus the sole cellular source of SPH is through the hydrolysis of ceramide; a reaction catalyzed by ceramidases [34-36]. Acid ceramidase has a pH optimum of approximately 4.5, thus the hydrolytic activity of this enzyme is thought to occur within lysosomes and/or late endosomes, whereas neutral and alkaline ceramidases are active at pH values ranging from 6.5 to 9.0 [54]. Studies using cloned neutral and alkaline ceramidases have shown that these enzymes can catalyze both the forward and reverse reactions (ceramide degradation and synthesis) *in vitro* [55-57]. The neutral ceramidase is membrane-bound; however, it has yet to be determined whether this enzyme is associated with the nuclear membrane. Of note, studies have shown that PA and phosphatidyl serine stimulate the hydrolytic activity of neutral ceramidase [58]. Based on our current findings, it is plausible that PA may serve as a feedback inhibitor of SF1-dependent transcription by stimulating the production of SPH, the inhibitory ligand.

Scintillation proximity assays demonstrated that several phospholipids and sphingolipids compete with radiolabeled SPH for binding to SF1 (Figure 9). Of the lipids that displaced SPH from the ligand binding pocket, only PA significantly induced SF1-dependent CYP17 reporter gene activity. Further, studies using chemical inhibitors and RNAi found that the enzyme important for PA production in H295R cells is DAGK-θ. DAGK-θ has been found localized in speckle domains of nuclei from various cell lines [59]. Nuclear DAGK-θ co-localized with $PI(4,5)P_2$, suggesting coupling of PIP_2 hydrolysis and PA formation [59]. Both DAGK-α [60] and DAGK-θ [44] are regulated by PI3K. Our findings that silencing PI3K protein expression significantly reduces

cAMP-stimulated CYP17 mRNA expression (Figure 18) support a relationship between DAGK and PI3K. Studies are underway to characterize the mechanism by which cAMP/PKA regulate PI3K activity in H295R cells. Interestingly, SPH increases nuclear PA formation, likely by stimulating DAG kinase activity [61]. In light of our current findings, we propose that SPH and PA may reciprocally activate the synthesis of each other. In the presence of cAMP, the conformation of SF1 is changed to allow dissociation of SPH. Once dissociated from SF1, SPH then acts to augment DAG kinase activity, thereby increasing production of PA, which in turn stimulates SF1-dependent transcriptional activity. To terminate SF1-dependent transcription, PA may act as a feedback inhibitor by stimulating ceramidase activity. Using LC-ESI-MS/MS, we have also found that PA is bound to SF1 purified from H295R cells (unpublished data), however further studies are needed to quantify the amounts of PA bound to the receptor and to determine the effect of cAMP on the amounts of PA in nucleus.

As previously discussed, both recently published crystal structures have revealed that SF1 contains a large ligand binding pocket which is filled by a phospholipid ligand [23, 24]. Li *et al.*, show that the interaction of SF1 with coactivators is reduced both by the absence of phospholipids and by phospholipids with longer fatty acids, longer acyl chains are predicted to project out of the pocket and interfere with the folding of the AF-2 helix into the active conformation [24]. Although both studies identified different phospholipids in the SF1 ligand binding pocket, neither study showed the presence of SPH. This is because bacteria do not synthesize sphingolipids. Further, in both structural studies, bacterially expressed SF1 was used to identify the phospholipid ligands, whereas in the present study, we immunoprecipitated SF1 from H295R adrenocortical cells and

subjected the purified receptor to analysis by LC-ESI-MS/MS. Thus, our findings provide physiologically relevant confirmation of the recently published crystallographic studies. Additionally, our findings demonstrate a mechanism by which cAMP can activate the exchange of an inhibitory ligand (SPH) for a stimulatory ligand (PA). As the ligand binding pocket of SF1 is large, it is likely that SF1 has multiple ligands. Our scintillation proximity assay data showing that several phospholipids can compete with SPH for binding suggest that SF1 may respond to different lipids in a context-specific manner to direct the expression of genes involved in development and steroidogenesis.

Our findings, showing cAMP-stimulated decreases SPH binding to SF1 (Figure 7) are in agreement with the prediction of Li *et al.*, that SF1 readily exchanges its ligands [24]. It is plausible that cAMP alters lipid amounts in distinct organelles (such as the nucleus) and that these changes are essential for steroidogenic gene transcription. Studies are underway to determine the effect of cAMP on the subcellular populations of phospholipids and sphingolipids. In summary, our findings demonstrate a mechanism by which SPH and PA act in a reciprocal manner as inhibitory and stimulatory ligands for SF1, respectively. We propose that ACTH/cAMP activates CYP17 in the human adrenal cortex by activating DAGK-θ, thus regulating this differential binding of SPH and PA to SF1.

APPENDIX A

PROTOCOLS

Immunoprecipitation (IP)

1. Treat for appropriate time with agent/stimulus
2. Wash cells twice with 8 ml PBS
3. Add 1 ml RIPA buffer with 1X protease inhibitors
4. Scrape plate into clean microfuge tube
5. Incubate on ice for 30 minutes
6. Sonicate 10 times for 5 seconds each , keep samples cold at all times
7. Spin at full speed for 15 minutes at 4ºC
8. Transfer supernatant to a clean tube and pre-clear by adding:
9. 20 µl protein A/G agarose
10. 1 µg of IgG (If primary antibody is rabbit then use rabbit IgG. If antibody is raised in mouse, use mouse IgG)
11. Rotate for 30 minutes at 4ºC
12. Spin at full speed for 15 minutes at 4ºC
13. Transfer supernatant to a clean tube and add:

    1-10 µl primary antibody (SF1, PP2A, etc)
    25 µl protein A/G agarose
    10µl BSA (20mg.ml)

14. Rotate overnight at 4ºC
15. Spin at 4000 rpm for 5 minutes
16. Remove supernatant and wash beads for 5 minutes
    2x with RIPA buffer
    2x with PBS

17. Add 30 µl SDS loading dye to each sample

18. Boil 5 minutes, cool on ice for 5 minutes

19. Load samples onto SDS/PAGE gel

SDS Poly-Acrylamide Gel

1. Resolving Gel (10 %, 10 ml):
   a. 4.0 ml H_2O
   b. 3.3 ml 30 % acrylamide
   c. 2.5 ml 1.5 M Tris, pH 8.8
   d. 0.1 ml 10 % SDS
   e. 0.1 ml 10 % ammonium persulfate
   f. 0.004 ml TEMED
2. Load gel.
3. Pour 30 % ethanol on top of gel
4. Stacking Gel (5%, 4 mL):
   a. 2.7 ml H_2O
   b. 0.67 ml 30 % acrylamide
   c. 0.5 ml 1.5 M Tris, pH 6.8
   d. 0.04 ml 10 % SDS
   e. 0.04 ml 10 % ammonium persulfate
   f. 0.004 ml TEMED
5. Remove the ethanol, load the stacking gel, and put the combs in.

Sample Preparation

1. Prepare loading buffer:
   2X SDS gel loading buffer: 1M DTT:: 9: 1
2. Prepare a mixture of equal volumes of loading buffer and samples depending on the size of the wells (small:15-20 µL per well, large:30-40 µL per well)
3. Heat samples at 100^{o}C (incubator ~4) for 5 minutes. Put a hole on top of each tube.
4. Place tubes on ice.

Running the Gel

1. Use protein markers.
2. Use 1X SDS-PAGE buffer.
3. Run the gel at 50-60 V.
4. When the bands reach the resolving gel, turn the voltage up to 100-120 V.

Transferring the Gel

1. Use Western Blot Buffer (100 mL):
    a. 0.025 M Tris base (2.5 mL 1M Tris, pH 8)
    b. 0.192 M glycine (1.441 g)
    c. 20 % methanol (20 mL)
    d. pH 8.3
2. Place three pre-soaked sheets of filter paper on bottom.
3. Place membrane soaked in methanol on top.
4. Place three additional sheets of filter paper on top.
5. Transfer the gel at 100 mA for 30 minutes.
6. Place membranes in 5% milk in PBS-Tween for blocking overnight.

Western Blotting

All at room temperature

I. Electrophoresis and blotting
- Pre-treat PVDF membrane
- In methanol for 5 second
- In water for 5 minutes
- In transfer buffer for 10-15 minutes
- Separate proteins using SDS-PAGE electrophoresis and electroblotting on to Hybond P (PVDF) membrane

II. Blocking Membrane
- Immerse membrane in 5% (w/v) blocking agent in PBS-T for one hour on orbital shaker at room temperature

III. Washing
- Dilute primary antibody in PBS-T
- On orbital shaker wash twice for 2 minutes with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 15 minutes each with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 5 minutes each with large volume of PBS-T (fresh washing buffer in-between)

IV. Incubation
- Incubate membrane in diluted primary antibody for 1 hour on orbital shaker

V. Dilution of fluorecein-linked anti-species antibody
- Dilute fluorescein-linked anti-mouse Ig for pERK or anti-rabbit Ig for ERK 1:2000 in PBS-T

VI. Washing
- On orbital shaker wash twice for 2 minutes with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 15 minutes each with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 5 minutes each with large volume of TBS-T or PBS-T (fresh washing buffer in-between)

VII. Incubation
- Incubate membrane in diluted secondary antibody for 1 hour on orbital shaker

VIII. Dilution of Anti-fluorescein alkaline phosphotase conjugate

- Dilute anti-fluorescein AP conjugate 1:2500 with PBS-T

IX. Washing

- On orbital shaker wash twice for 2 minutes with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 15 minutes each with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 5 minutes each with large volume of PBS-T (fresh washing buffer in-between)

X. Incubation

- Incubate membrane in diluted conjugate for 1 hour on orbital shaker at room temperature

XI. Washing

- On orbital shaker wash twice for 2 minutes with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 15 minutes each with large volume of PBS-T (fresh washing buffer in-between)
- On orbital shaker wash 2 times for 5 minutes each with large volume of PBS-T (fresh washing buffer in-between)

XII Cover the membrane with ECF and incubate for 20 mins.

XIII Let the excess ECF drain, and then incubate the membrane in dark for 20min.

XIV Develop the membrane using phosphoimager.

Trizol RNA Preparation

Wash cells 2x with PBS

Add 500µl Trizol to each well and scrape into 1.5ml tubes

Incubate at room temperature for 5 minutes

Add 100µl chloroform/ iso amy

Shake for 20 seconds

Incubate at room temperature for 3 minutes

Spin at 12,000 rpm for 15 minutes at 4ºC

Remove aqueous phase (clear) to new tube

Add 200µl isopropanol

Incubate at room temperature for 10 minutes

Spin at 12,000 rpm 4ºC for 10 minutes

Remove supernatant and add 500µl 75% ETOH

Vortex briefly and spin at 12,000 rpm 4ºC for 5 minutes

Remove ethanol

Air dry 15-30 minutes at room temperature

Resuspend in 25 µ**l** DEPC-H2O

siIMPORTER Protocol For RNA Interference

1. change Media according to Table 1

Table 1:

FORMAT	MEDIA (µl)	siRNA/siIMPORTER (µl)	TOTAL (µl)
12 well	465	35	500
6 well	930	70	1000

2. in microcentrifuge tude add siIMPORTER and RPMI based on Table for a whole 12 well plate:

   siIMPORTER vol. = 2.5 • 12 wells = 30 µl
   RPMI = 12.5 • 12 wells = 150 µl

Table 2:

	PER WELL	
FORMAT	**SiIMPORTER (in µl)**	**RPMI (in µl)**
12 WELLS	2.5	12.5
6 WELLS	5	25

3. mix by pipetting

4. in another microcentrifuge tube mix diluent, RPMI and siRNA according to table 3

   (this will give a 100 nM/ well)

Table 3:

FORMAT	Diluent (µl)	RPMI (µl)	siRNA (µl)
12 well	12.5	5	2.5
6 well	25	10	5

5. mix by pipetting

6. add step 4 to step 2

7. incubate for 5-10 mininutes and add 35µl dropwise to each well

Site Directed Mutagenesis

PCR reactions: 50ul run

10x Pfu buffer
10 mM dNTP's
DMSO
50ng template
125 ng forward primer
125 ng reverse primer
H_20
Turbo

Cycles: x16

30 sec @ 95ºC
1 min @ 55ºC
10 minutes @ 68ºC

Add 2µl Dpn1 to reaction and incubate @ 37ºC for 1 hour

Perform gel electrophoresis on 20µl of sample.

Transformation:

Add 2µl (or more if necessary) PCR reaction to 50µl XL1 blue cells
Incubate on ice for 30 mininutes
Heat shock at 42ºC for 1 minutes
Incubate on ice for 5 minutes
Add 500µl LB
Incubate @ 37ºC for 1 hr with constant shaking
Plate on selective media
Select colonies & grow up overnight
Determine if mutation occurred by sequencing

Luciferase Assay

Wash cells twice with PBS

Remove PBS completely

Prepare Passive Lysis Buffer (Promega) by Diluting 5x stock with H_2O

(1 part 5x: 4 parts Water)

Add 100µl Passive Lysis Buffer to each well

Place plates in –80ºC for 10-20 minutes

Place plates in 37 degree water bath for approximately 1 minute (until contents in all wells have melted)

Repeat freeze/thaw cycle twice

Thaw appropriate volumes of Dual Luciferase Assay (Promega) and Stop and Glo Reagents

Luciferase Assay Reagent 100ul/sample

Stop and Glo Reagent 100ul/sample

(Dilute Stop and Glo Substrate 1 part: Stop and Glo Buffer 49 parts)

Read RLU using Luminometer

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